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Isolation and Comparative Biochemical Properties of the Major Internal Polypeptides of Equine Infectious Anemia Virus

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We describe procedures for the large-scale production of equine infectious anemia virus (EIAV) and for the isolation of the four major non-glycosylated virion proteins, designated p26, p15, p11, and p9. Comparisons of the purified proteins by peptide mapping procedures and by enzyme-linked immunosorbent assays demonstrated the unrelatedness of the four proteins. The characteristic properties of each purified protein were examined by determining isoelectric points and amino acid compositions. We found that EIAV p26 and p9 focus at pI values of 6.2 and 5.0, respectively, and that these proteins contain no unusual amino acids. In contrast, EIAV p15 reproducibly displayed a heterogeneous isoelectric focusing pattern, with major pI values ranging from 5.7 to 8.3. This charge variation evidently correlated with different levels of phosphorylated serine or threonine or both, which could be detected by an amino acid analysis of purified p15. EIAV p11 apparently focused at a pI of >10, reflecting its high content of basic amino acids. Moreover, localization experiments indicated that all four nonglycosylated proteins constitute the internal components of the virus, with all of the virion p11 closely associated with the viral RNA genome. Thus, our results demonstrated that EIAV, a lentivirus, contains structural polypeptides which are analogous to the structural polypeptides described previously in prototype C oncoviruses.

Equine infectious anemia is unique among virus-induced diseases in that clinical symptoms and associated bursts of viremia occur in sequential episodes separated by several weeks or months (13, 23). The available evidence indicates that the periodic nature of this disease is due to the sequential production and release of novel antigenic strains of equine infectious anemia virus (EIAV) which temporarily escape host immunosurveillance systems (13, 23–26). Thus, EIAV provides a dynamic system for investigating the interaction between host immune mechanisms and antigens involved in a persistent virus infection. However, a prerequisite for studying this system is a thorough description of the antigens of the causative virus.

EIAV matures by budding from cytoplasmic membranes (31, 48), displays a complex morphology characteristic of type C viruses (16), and contains a reverse transcriptase (2) and a high-molecular-weight (60S to 70S) RNA genome composed of subunits (11). Based on these properties, EIAV has been tentatively classified as a member of the family Retroviridae (9, 13, 23, 46). However, serological comparisons have failed to detect any relatedness between EIAV and a number of retroviruses (9, 46), and some ultrastructural studies (16) have suggested that EIAV resembles visna virus, a member of the lentivirus subfamily, more closely than any member of the oncovirus subfamily. These observations and the initial descriptions of EIAV and visna virus polypeptides have led to the suggestion that the protein composition of lentiviruses may be distinct from the protein compositions of prototype C mammalian oncoviruses (7, 10, 13, 22, 27, 42). Moreover, this proposed difference in polypeptide content has been suggested as a distinguishing feature between the oncovirus and lentivirus subfamilies of the Retroviridae (30).

We recently presented evidence that EIAV contains structural polypeptides analogous to those found in murine and avian type C oncoviruses, including two glycoproteins (gp90 and gp45) and four major non-glycosylated proteins (p26, p15, p11, and p9) (38). Because of the limited amounts of virus produced in standard tissue culture systems, analytical studies of EIAV polypeptides have been limited (9, 10, 22, 38). We have now greatly improved the production of virus in tissue culture, making feasible

the isolation of virion structural proteins in quantities sufficient for biochemical and serological analyses. In this paper we describe procedures for the isolation of the internal polypeptides of EIAV and their biochemical characterization. Our results support the proposal that EIAV contains polypeptides analogous to those of classical oncoviruses. We also provide a catalog of the key properties of the internal proteins of EIAV, including isoelectric points, amino acid compositions, peptide maps, and serological properties.

MATERIALS AND METHODS

Virus propagation and purification. The cell-adapted Wyoming strain (29) of EIAV was propagated in primary equine fetal kidney cells maintained in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5 to 10% sterile newborn calf serum (GIBCO), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 10 mM sodium bicarbonate, 150 U of penicillin G per ml, and 150 μg of streptomycin sulfate per ml. Persistently infected cultures were maintained in roller bottle cultures containing 50 ml of medium. Under these conditions virus production continued for approximately 3 months, until cell senescence.

Supernatant medium was harvested every 3 to 4 days for virus purification. After the cell debris was removed from the medium by centrifugation at 8,000 rpm for 30 min in a Sorvall GSA rotor, the virus-containing supernatant was concentrated about 10- to 40-fold by using a Pellicon membrane filter cassette system (nominal exclusion, 10^6 molecular weight; Millipore Corp., Bedford, Mass.). The virus concentrate was then layered over a 10-ml cushion of 10% sucrose, and the virus was pelleted by centrifugation in a type 19 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 19,000 rpm for 2 h. The pelleted virus was suspended in 10 mM sodium phosphate buffer (pH 7.2) and sedimented to its equilibrium density (1.18 g/ml) on 3-ml 20 to 80% (vol/vol) glycerol gradients in a Beckman SW50.1 rotor centrifuged at 45,000 rpm for 2 h. All procedures were carried out at 4°C.

With these procedures, about 10 mg of EIAV protein (as determined by the procedure of Lowry et al. [28]) could be purified per week from 10 roller bottles yielding 1 liter of tissue culture fluid. Previously, other techniques in which equine dermal cells were used (1, 3) produced only 1 mg of EIAV per 10 roller bottles per week. The improved virus yield is evidently a result of the kidney cell system since identical purification procedures were used to isolate virus from the equine dermal cell system (1).

Protein isolation. The procedures used for sample preparation and the conditions used for guanidine hydrochloride gel filtration were based on the methods of Green and Bolognesi (17) and Montelaro et al. (34). As reported previously (38), routine procedures for solubilizing retrovirus protein samples in guanidine hydrochloride do not completely dissociate the proteins of EIAV, some of which chromatograph together in the void volume as aggregates. However, it has been determined that EIAV proteins can be resolved more efficiently if the virion lipids are extracted with acetone before the proteins are treated with guanidine hydrochloride (32). Thus, in a typical experiment about 30 mg of purified EIAV in 4 ml of 10 mM sodium phosphate buffer (pH 7.2) was mixed with 10 volumes of cold acetone and incubated at 4°C for 45 min with frequent mixing. The resulting protein precipitate was collected by centrifugation in a Sorvall SS-34 rotor at 8,000 rpm for 30 min, and the supernatant acetone phase containing viral lipids was removed. The protein pellet was then solubilized in 4 ml of 8 M guanidine hydrochloride in 50 mM Tris buffer (pH 8.5) containing 2% 2-mercaptoethanol by heating in a boiling water bath for 5 min and at 45°C for 45 min. Any insoluble material was removed by another centrifugation, but usually quantitative solubilization of the starting virus protein was achieved.

The solubilized proteins were then analyzed on a column (1.5 by 95 cm) of Sepharose 6B (Pharmacia Chemical Co., Uppsala, Sweden) eluted with 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5) at a flow rate of 1.2 to 1.6 ml/h. The recommended sodium acetate buffer (17) (pH 5.0) was not used because of the rapid degradation of the Sepharose matrix at this pH. The absorbance at 280 nm was measured by a continuous flow monitor, while fractions of about 0.5 ml were collected. The appropriate fractions were pooled, dialyzed for 48 h against several changes of 100 mM ammonium bicarbonate, and finally dialyzed against 50 mM bicarbonate for 24 h. The dialyzed samples were lyophilized to dryness and suspended in an appropriate buffer. A recovery of 60 to 70% of the initial virus protein was usually observed at this stage of the purification.

To remove minor amounts of cross-contaminating proteins from p11 and p9 derived from guanidine hydrochloride gel filtration, these preparations were further purified by ion-exchange chromatography on a column (1 by 6 cm) of phosphocellulose (Whatman P11) eluted stepwise with 10-ml volumes of buffer containing 0 to 1 M KCl in 0.1 M increments (47). The p9 component eluted in the buffer containing no KCl, whereas p11 bound tightly to the resin and eluted only with 0.7 M KCl. Thus, the ion-exchange procedure was effective in separating p11 and p9. EIAV p26 and p15 coeluted from the resin at salt concentrations of 0.3 to 0.4 M. This binding property ensured that any p26 or p15 contaminating p9 or p11 was effectively removed during the stepwise elution procedure. However, the coelution of p26 and p15 precluded the use of phosphocellulose as a further purification step for the p26 and p15 derived from guanidine hydrochloride gel filtration. Therefore, these two proteins were subjected to a second separation by guanidine hydrochloride gel filtration, as described above.

The radioactive virion proteins used in peptide mapping and isoelectric focusing studies were isolated directly from a single guanidine hydrochloride gel filtration run, as described previously (38).

SDS-PAGE. The procedures used for sample preparation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on slab or cylindrical polyacrylamide gels, and analysis of gels by staining or fluorography have been described previously (33, 43, 44). However, the gels used in this study contained 1 M urea, which enhanced the resolution of the virion

low-molecular-weight proteins compared with the standard gel system. Protein molecular weight calibration standards were obtained from Pharmacia.

Isoelectric focusing on polyacrylamide gels. Protein isoelectric points were determined by using cylindrical polyacrylamide gels (0.6 by 10 cm) containing 7.5% acrylamide, 0.2% bisacrylamide, 5 M urea, and 1.6% ampholine (pH range, 3.5 to 10; LKB, Munich, Germany). Protein samples were dissolved in deionized water containing 5 M urea and 10% sucrose. Electrolyte solutions consisted of 1% ethylenediamine at the anode and 1.4% phosphoric acid at the cathode. Electrophoresis was carried out at 4°C at constant power until less than 1 mA of current was observed (usually 10 to 12 h). Gels were then fractionated into 1-mm samples with an automatic fractionator (Gilson Medical Electronics, Middleton, Wisc.), and the fractions were assayed for pH and radioactivity (34).

Peptide mapping. Purified, homogeneous preparations of [^{3}H]leucine-labeled EIAV p26, p15, p11, and p9 were subjected to limited proteolysis by chymotrypsin or *Staphylococcus aureus* protease V8, and the resulting peptides were separated by SDS-PAGE on a 15% polyacrylamide slab gel (12). Typically, about 2×10^4 dpm of purified protein was treated for 2 h with either 0.65 U of chymotrypsin or 5 U of protease V8 in a total reaction volume of 0.1 ml. Radioactive, leucine-containing peptides were detected by standard fluorographic techniques (43).

Amino acid analysis. Samples of purified EIAV p26, p15, p11, and p9 were hydrolyzed in 6 N hydrochloric acid at 110°C for 24 h in sealed, evacuated ampoules containing a crystal of phenol to prevent destruction of tyrosine (19). Hydrolyzed samples were analyzed with a Beckman model 119 automatic amino acid analyzer equipped with an automatic sample injector. The analytical conditions used were essentially the conditions described previously (4) for single-column methodology. Colorimetric response factors were determined by using standard amino acid mixtures (Pierce Chemical Co., Rockford, Ill.). The analytical conditions used for phosphoserine and phosphothreonine measurements were determined by using authentic samples of the amino acids (Sigma Chemical Co., St. Louis, Mo.); the optimum hydrolysis time was determined to be 5 h, as recommended previously (17, 36). The tryptophan content was estimated by the nondestructive, spectroscopic procedure of Edelhoch (15).

ELISA. Enzyme-linked immunosorbent assays (ELISA) of antisera produced in rabbits to purified p26, p15, p11, and p9 and to purified EIAV were conducted in micro-ELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.) by using commercially available goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma Chemical Co.). For these comparative tests approximately 200 ng of each purified antigen was applied to each well. Approximately 10% antigen binding was observed after overnight incubation, as measured with radioiodinated EIAV p26. Standard procedures were followed, with minor kinetic modifications (41).

Production of virion subparticles. Virion surface and internal proteins were differentiated based on their susceptibilities to protease digestion when intact, [^{3}H]leucine-labeled EIAV was treated with bromelain, and the resulting subparticle polypeptide composition was analyzed by SDS-PAGE, using the procedures described by Mosser et al. (35). The virion ribonucleoprotein complex was produced by treating purified EIAV with Nonidet P-40 (Shell Chemical Co., St. Louis, Mo.), purified by centrifugation on a sucrose gradient, and analyzed for protein composition by SDS-PAGE (14).

RESULTS

Isolation of major polypeptides of EIAV. Figure 1 shows a representative absorbance profile obtained from a guanidine hydrochloride gel filtration analysis of about 30 mg of EIAV protein prepared and chromatographed as described above. Figure 2 shows the results of an SDS-PAGE analysis of the absorbance peaks revealed by guanidine hydrochloride gel filtration. An examination of the profiles indicated that guanidine hydrochloride gel filtration pools D, E, and G contained predominantly EIAV p26, p15, and p9, respectively. These results correspond to the results obtained previously with [^{3}H]leucine-labeled EIAV (38). However, guanidine hydrochloride gel filtration pool F (Fig. 1), which displayed negligible absorbance at 280 nm, contained the p11 component of EIAV (Fig. 2). This lack of absorbance was unexpected, but correlated with the absence of tyrosine and tryptophan in hydrolysates of p11 (see below).

A second unexpected result was the absence of detectable protein in the SDS-PAGE analysis (Fig. 2) of guanidine hydrochloride gel filtration pool A, which corresponded to the void volume fractions of the column (Fig. 1). In previous experiments with [^{3}H]leucine-labeled EIAV, all of the viral gp45 and significant quantities of p26 and p15 eluted in the void volume fractions,

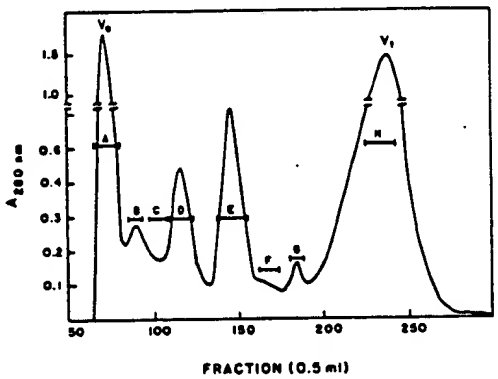

FIG. 1. Chromatographic separation of EIAV proteins by guanidine hydrochloride gel filtration as described in the text. The fractions indicated were pooled and analyzed by SDS-PAGE (see Fig. 2). V_o indicates the void volume fraction of the column determined with blue dextran, and V_t indicates the total column volume measured with dinitrophenylalanine. A_{280nm}, Absorbance at 280 nm.

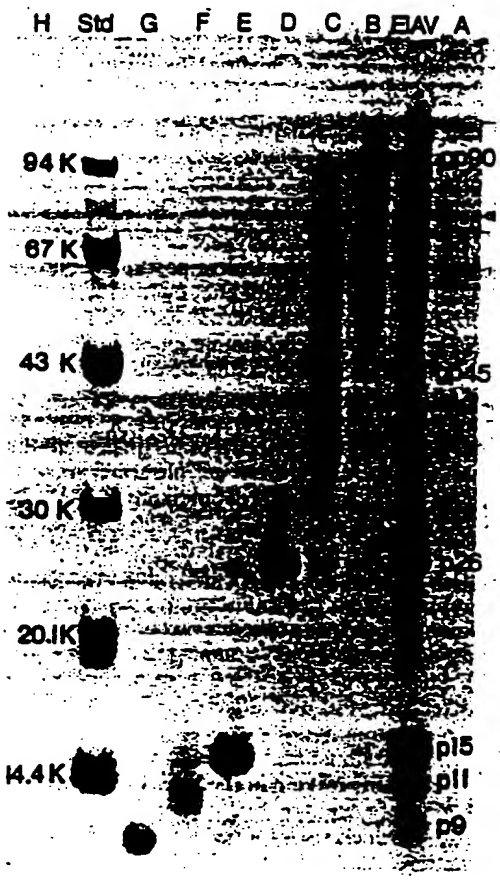

FIG. 2. SDS-PAGE analysis of proteins resolved by guanidine hydrochloride gel filtration. The protein pools tested (as designated in Fig. 1) are indicated above the lanes. Purified EIAV and protein standards (Std) were included as references; the molecular weights are indicated on the left (94K, molecular weight of 94,000). About 20 μg of each protein pool, 75 μg of EIAV, and 60 μg of the protein standard mixture were prepared for and analyzed by SDS-PAGE as described previously (34, 43, 44).

evidently as aggregates (38). Figure 2 shows that EIAV gp45 was present in guanidine hydrochloride gel filtration pool C and that p26 and p15 were found only in pools D and E, respectively. Along with a number of other minor proteins of unknown significance, EIAV gp90 was present in pool B, whereas the final peak of absorbance, (pool H) contained no proteins detectable by SDS-PAGE (Fig. 2). The absorbance in pool H (Fig. 1) appeared to be due to mercaptoethanol, which absorbs at 280 nm and was present in the disruption buffer, and to light diffraction by residual virion lipids. The chromatographic behavior of the EIAV proteins was confirmed by a parallel guanidine hydrochloride gel filtration analysis of acetone-treated [^{3}H]leucine-labeled EIAV and purified [^{3}H]glucosamine-labeled gp90 and gp45 (data not shown). Thus, it appears that the reduction of viral lipid content by acetone treatment prevents the aggregation of EIAV proteins and ensures the complete dissociation of these proteins to monomers in guanidine hydrochloride.

Although the SDS-PAGE analysis shown in Fig. 2 indicated that EIAV p26, p15, p11, and p9 were isolated essentially as homogeneous preparations, minor quantities (<2%) of neighboring proteins could be detected if gels were heavily overloaded or if the respective protein pools were radioiodinated by the chloramine T procedure. These minor contaminants in p26 and p15 were removed by a second purification, using guanidine hydrochloride gel filtration. EIAV p11 and p9 were further purified by phosphocellulose ion-exchange chromatography, in which p9 had no affinity for the resin but p11 bound strongly to the column and eluted only with high concentrations of salt (see above). When these procedures were used, all of the isolated virion proteins displayed a single, homogeneous band of stain or peak of radioactivity in SDS-PAGE analyses of unlabeled or radioiodinated protein samples (data not shown). It is important to note that EIAV p11 cannot be labeled by the chloramine T radioiodination procedure, evidently because of the lack of tyrosine residues in the protein (see below). EIAV p26, p15, and p9 displayed typical iodination efficiencies of 1×10^7, 2.3×10^6, and 6×10^7 dpm/nmol, respectively.

Peptide mapping. Previous reports from other laboratories (10, 13, 22) have described fewer than the four major non-glycoslylated EIAV proteins reported by our laboratory. Thus, we sought to establish conclusively whether the four virion proteins resolved by our analytical procedures are unique or share amino acid sequences, perhaps as cleavage products of each other. To do this, homogeneous preparations of [^{3}H]leucine-labeled p26, p15, p11, and p9 were subjected to limited proteolysis by chymotrypsin or *S. aureus* protease V8, and the resulting peptides were analyzed by SDS-PAGE, as described by Cleveland et al. (12). The autoradiographs in Fig. 3 show that the fragments produced by protease V8 treatment of p26, p15, and p11 are unique; p9 appeared to be digested rapidly to small fragments which migrated from the gel. In a complimentary manner, the SDS-PAGE analysis (Fig. 3) revealed novel chymotrypic peptides obtained from p26, p15, and p9; p11 was apparently cleaved to small fragments which migrated from the gel. The unique peptides generated by the two proteases and the

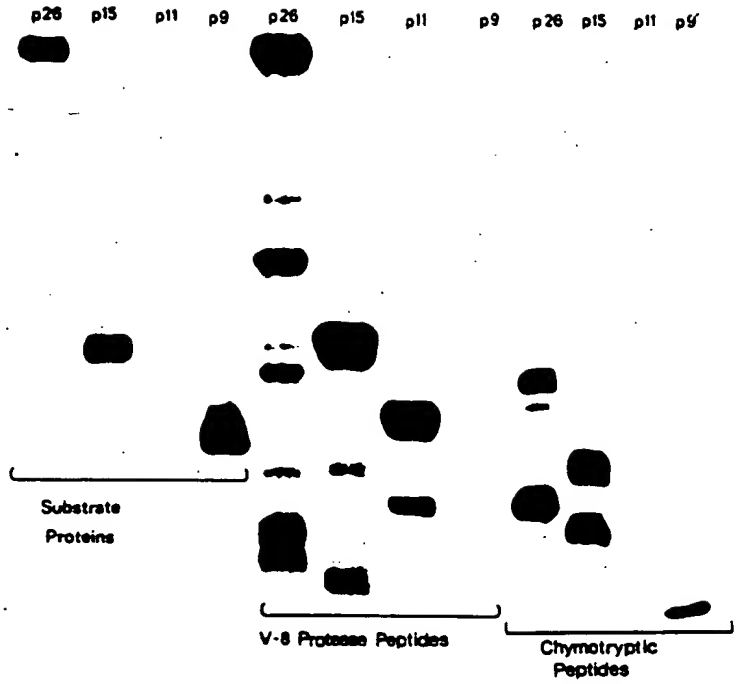

FIG. 3. SDS-PAGE analysis of peptide fragments generated by limited proteolysis of samples of [^{3}H]leucine-labeled EIAV p26, p15, p11, and p9, using either *S. aureus* protease V8 or chymotrypsin. Untreated radioactive EIAV proteins were included for reference.

characteristic efficiencies of cleavage indicate that EIAV p26, p15, p11, and p9 contain unique amino acid sequences and therefore are not related. This conclusion is further supported by the characteristic chemical and serological properties of each protein, as described below.

Isoelectric points. The isoelectric point of each protein was determined by subjecting [^{3}H]leucine-labeled preparations of p26, p15, p11, and p9 to isoelectric focusing on polyacrylamide gels, as described above. Each protein preparation displays a single homogeneous peak of radioactivity upon analysis by SDS-PAGE (38). The electrophoretograms in Fig. 4 show single homogeneous peaks of radioactivity for EIAV p26 (Fig. 4A) (pI, 6.2) and p9 (Fig. 4C) (pI, 5.0). EIAV p11 migrated toward the cathode as a single species, but eventually migrated from the gel, indicating a pI of >10.0 (data not shown).

In contrast to the single isoelectric species described above, EIAV p15 reproducibly focused in a characteristic heterogeneous pattern, with major portions of the protein appearing at pI values of 5.7, 6.6, 7.6, and 8.3 (Fig. 4B). This heterogenous pattern varied only slightly when p15 was isolated from different virus preparations. The phosphoproteins of avian and mammalian oncoviruses have been shown to display a similar charge heterogeneity, reflecting different degrees of protein phosphorylation (18). Thus, our results suggest that p15 may be a phosphoprotein component of EIAV.

Amino acid compositions. The availability of sufficient quantities of homogeneous samples of p26, p15, p11, and p9 made feasible accurate determinations of the amino acid compositions of these proteins by standard procedures. Table 1 shows amino acid compositions similar to those reported for avian and mammalian oncoviruses (19, 36). For example, like murine virus p10 or avian virus p12, EIAV p11 contains a high content (17%) of the basic amino acids lysine and arginine, as well as a high content of glycine (15%). High levels of these amino acids have been correlated with the highly basic and flexible nature of the ribonucleoproteins of avian and murine oncoviruses (19, 36) and of histones, which suggests a similar role for EIAV p11. Also, p11 contains no tyrosine residues, which explains its resistence to chloramine T-mediated iodination, and lacks tryptophan, which correlates with the negligible absorbance of p11 at 280 nm.

Hydrolysates of EIAV p9 revealed 16 residues of glutamate and 8 residues of aspartate (a total of 28% of all residues). Although the amide proportion of these amino acids was not determined, the low isoelectric point (pI, 5.0) of the

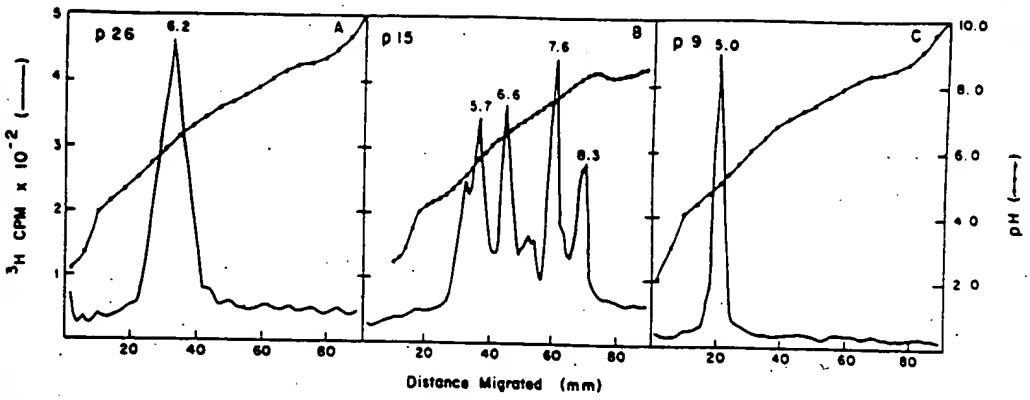

FIG. 4. Isoelectric focusing of [^{3}H]leucine-labeled proteins by electrophoresis on cylindrical polyacrylamide gels. The procedures used for sample preparation and the electrophoresis conditions are described in the text. The proteins and their isoelectric point(s) are indicated.

protein even though it contains nine lysine and two arginine residues per molecule suggests that most of the glutamic and aspartic residues are not amidated. Also, the high susceptibility of p9 to digestion by protease V8 can be explained by the high content of glutamic acid residues.

The heterogeneous pattern observed during our analysis of p15 by isoelectric focusing suggests the presence of phosphoserine or phosphothreonine or both in this protein. However, the phosphorylated amino acids undergo complete hydrolysis to serine and threonine during the standard 24-h acid treatment used and thus cannot be detected upon subsequent amino acid analysis. To assay for the presence of phosphoserine or phosphothreonine, samples of p15 were subjected to a 5-h hydrolysis protocol which did not destroy phosphoamino acids (37), and the resulting hydrolysates were analyzed by standard procedures (Fig. 5). Our results indicated the presence of ninhydrin-positive material which eluted from the resin in the position of phosphoserine and phosphothreonine standards, which coeluted (Fig. 5A and B). This acidic component from p15 diminished progressively with longer acid hydrolysis and was completely removed after 24 h of hydrolysis (Fig. 5C). Similar kinetics were observed with phosphoserine and phosphothreonine standards. As a control, samples of purified lysozyme, which lacks phosphoamino acids, and of EIAV p26 were subjected to limited hydrolysis, and their amino acid contents were analyzed by standard procedures. Neither of these proteins displayed components that chromatographed in the position of the phosphoamino acids (Fig. 5D). Thus, our results indicated that EIAV p15 contains phosphoserine or phosphothreonine or both, although the relative abundance of each amino acid could not be determined by this method. However, attempts to incorporate $^{32}P_i$ into p15 by standard in vivo procedures (18, 37) have been unsuccessful, perhaps due to suboptimal labeling conditions for the equine kidney cells.

One final observation on the amino acid contents of the EIAV proteins is of practical concern. EIAV p11 and p9 contain no methionine residues, whereas p15 contains only one methionine; hence, radiolabeling of virus proteins with

TABLE 1. Amino acid compositions of EIAV proteins

Amino acid	No. of residues per mol[a]			
	p26	p15	p11	p9
Lysine	13	13	12	9
Histidine	3	2	3	2
Arginine	16	5	5	2
Aspartic acid	23	14	5	8
Threonine	14	10[b]	5	4
Serine	9	9[b]	6	9
Glutamic acid	34	20	13	16
Proline	14	3	8	2
Glycine	16	8	14	5
Alanine	16	9	7	3
Half-cysteine[c]	7	3	4	2
Valine	10	8	3	5
Methionine	7	1	0	0
Isoleucine	15	4	2	3
Leucine	18	14	7	8
Tyrosine	4	3	0	3
Phenylalanine	8	4	5	1
Tryptophan[d]	2	2	0	2

[a] Calculated by using molecular weights of 26,000, 15,000, 12,000, and 9,000 for p26, p15, p11, and p9, respectively (38). The total molecular weights of p26, p15, p11, and p9 were calculated to be 26,040, 14,870, 10,570, and 9,070, respectively, using the amino acid compositions presented.

[b] A portion of these residues appeared as phosphorylated amino acids upon analysis of 5-h hydrolysates.

[c] Determined as cysteic acid.

[d] Determined by spectroscopic analysis (15).

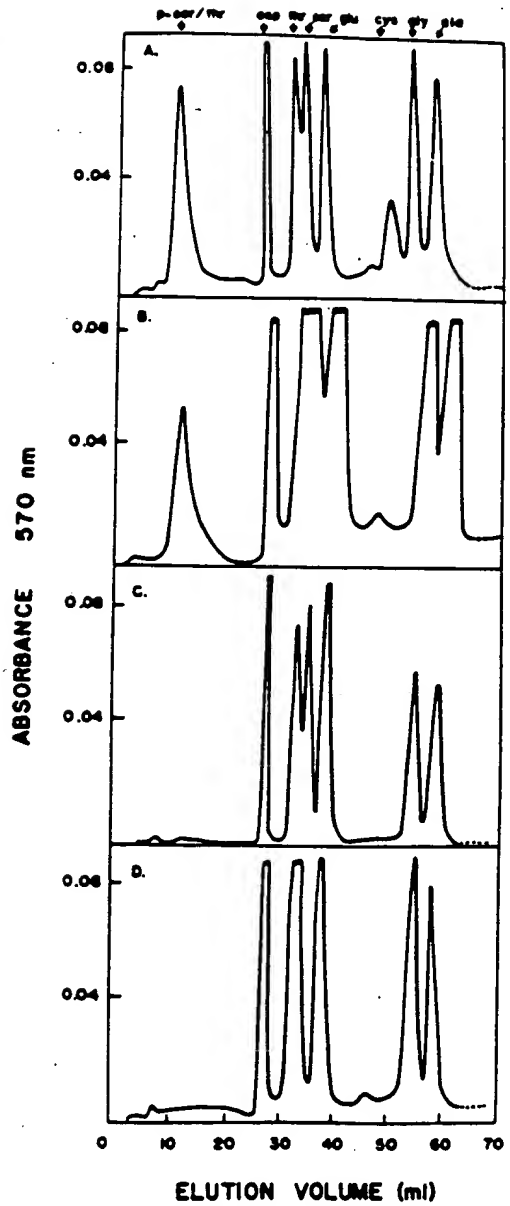

FIG. 5. Chromatograms from amino acid analyses of phosphoserine and phosphothreonine added to a standard amino acid mixture (A), EIAV p15 after 5 h of hydrolysis (B), EIAV p15 after 24 h of hydrolysis (C), and lysozyme after 5 h of hydrolysis (D). The same profile was observed for lysozyme after 24-h of hydrolysis. The procedures used for sample preparation and analysis are described in the text.

[^{35}S]methionine or peptide mapping by cyanogen bromide cleavage is not recommended.

Serological comparisons. The availability of homogeneous preparations of p26, p15, p11, and p9 permitted the production of antiserum against each viral protein and a serological comparison of the EIAV proteins. To do this, purified antigens and the resulting rabbit antisera were used in ELISA procedures (41) optimized for EIAV antigens. Table 2 shows that antiserum generated against disrupted EIAV displayed strong reactivities with all of the virion proteins. This indicated that the major viral antigens induced immune responses in the rabbits which were roughly proportional to the relative amounts of the antigens in the virions (38). In contrast to the broad specificity of the antiserum to disrupted EIAV, the sera produced against p26, p15, and p9 were reactive only in the homologous reaction; these sera showed no significant cross-reactivity. Only the antiserum produced against p11 demonstrated detectable cross-reactivity with p15. The reason for this minor cross-reactivity is not known; however, it is clear that p15 and p11 are not immunologically related since the anti-p15 serum failed to react with p11 antigen. Thus, our results indicate a lack of immunological relatedness, reflecting the unique biochemical properties of the EIAV non-glycosylated proteins.

Localization of virion proteins. According to the current model for oncovirus structure, the location of the structural polypeptides of virions can be correlated with certain biochemical properties (6, 33, 34, 45). To test this model with EIAV, the location of the virion polypeptides was determined by standard procedures in which purified virus was treated with protease (35) or detergent (14) and the resulting subparticles were isolated and analyzed for protein composition by SDS-PAGE.

The protease bromelain has been used routinely to distinguish surface proteins from proteins sequestered by the virion lipid bilayer from enzymatic digestion (35). Figure 6 shows that treatment of purified [^{3}H]leucine-labeled EIAV

TABLE 2. Comparative serological properties of EIAV proteins[a]

Test antigen	Reactivity with antiserum against:				
	p26	p15	p11	p9	EIAV
p26	20	0	0	0	14
p15	0	15	1	0	9
p11	0	0	4	0	5
p9	0	0	0	3	2

[a] Serological reactivities were determined by using ELISA procedures, purified antigen (about 200 ng), and rabbit antisera at dilutions of 1:1,000. Reactivities are expressed as arbitrary units, where 1 U represents an absorbance of 0.1 in the ELISA system. In control experiments, normal rabbit serum resulted in reactivities of less than 0.01 absorbance unit with all of the test antigens.

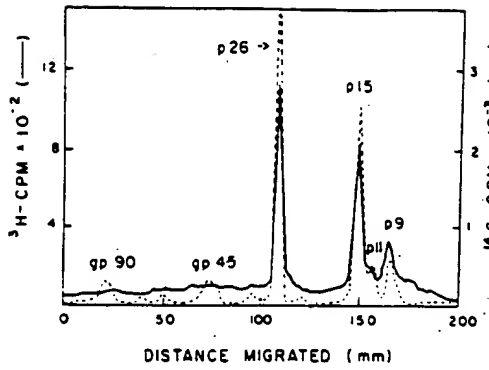

FIG. 6. SDS-PAGE analysis of bromelain-treated [^{3}H]leucine-labeled EIAV (solid line) and of untreated [^{14}C]leucine-labeled EIAV (dashed line) used as a reference.

with bromelain resulted in complete removal of the two viral glycoproteins (gp45 and gp90), whereas the major non-glycosylated proteins (p26, p15, p11, and p9) were not diminished by enzyme digestion. Although p15 and p11 were not resolved completely in the profile of bromelain-treated virus in Fig. 6, probably because of the comigration of some glycoprotein fragments in that region of the gel (34), the presence of p11 in addition to p26, p15, and p9 could be demonstrated by guanidine hydrochloride gel filtration analyses of bromelain-treated virus preparations (data not shown). In addition, bromelain digestion of [^{3}H]glucosamine-labeled EIAV, in which only gp90 and gp45 were radiolabeled (38), resulted in the complete loss of radioactivity from the virus particles. Thus, gp90 and gp45 appear to form the surface projections of the virus, whereas p26, p15, p11, and p9 constitute the internal virion components.

The identity of the internal protein(s) associated with the viral RNA was determined by treating purified EIAV with Nonidet P-40 to release the virion ribonucleoprotein complex, which was then isolated by centrifugation on a sucrose gradient (14). Typically, about 90% of the virion protein was solubilized by the detergent, and only 10% of the protein sedimented in the sucrose gradient in the position of the ribonucleoprotein subparticles. The results of SDS-PAGE analyses of the purified subparticles and of the virion proteins solubilized by the detergent are presented in Fig. 7. The SDS-PAGE profiles demonstrate that the ribonucleoprotein complex contains all of the detectable viral p11 and minor quantities of p26 and p15 (Fig. 7, lane A). An analysis of the detergent-solubilized protein fraction revealed most of the virion p26, p15, and p9; however, the p11 component was completely absent (Fig. 7, lane B). Also, all of the virion glycoprotein (gp90 and gp45) was solubilized by the detergent; negligible quantities were detected in the RNA particles. Thus, these results are similar to those observed with ribonucleoprotein particles isolated from avian (5, 14) and murine (5) oncoviruses, in which all of the basic internal protein is tightly bound to the viral RNA genome. The reproducible appearance of some (<10%) of the EIAV p15 and p26 with the ribonucleoprotein may reflect incomplete dissociation by the detergent or specific interactions of these proteins with the viral RNA or p11.

Figure 7 also shows the presence of several minor high-molecular-weight components (molecular weights, 30,000 to 70,000) that purified with the ribonucleoprotein particles (lane A) but were absent from the detergent-solubilized proteins (lane B). These minor virion proteins were

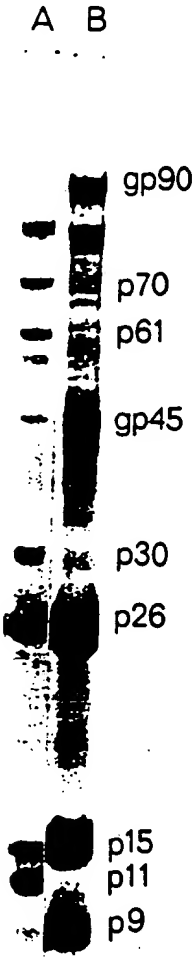

FIG. 7. SDS-PAGE analysis of the proteins solubilized by Nonidet P-40 treatment of purified EIAV (200 μg) (lane B) or contained in the isolated ribonucleoprotein complex (75 μg) (lane A).

detected previously in [^{3}H]leucine-labeled EIAV preparations, but their significance was unknown (38). By analogy to oncovirus systems, however, the location of these minor polypeptides exclusively in the ribonucleoprotein particles may suggest a role as components of the virion reverse transcriptase or as uncleaved polyprotein precursors of the internal proteins (33).

DISCUSSION

In this paper we describe procedures which accomplish the purification of the major non-glycosylated proteins of EIAV in quantities and homogeneities sufficient for detailed biochemical analyses. A useful development is the observation that the removal of virion lipids by acetone extraction before guanidine hydrochloride gel filtration eliminates the troublesome protein aggregation problem previously observed with EIAV (38) and with visna virus (27). It is curious that this problem has never been noted with avian or murine oncoviruses (34) but appears to be characteristic of lentiviruses. However, the role of lipid in enhancing protein aggregation in guanidine hydrochloride solutions may explain the apparent discrepancy in which certain investigators (21, 34) observed murine p15E aggregated in the void volume fractions from guanidine hydrochloride gel filtration columns, whereas others (20, 40) found that p15E cochromatographed with the p15C component as monomers. An examination of the procedures used by the various investigators reveals that those workers who used acetone extractions during sample preparation reported that p15E copurified with p15C, whereas those who observed p15E in the void volume omitted the lipid extraction step.

Some biochemical characterizations of EIAV and other lentiviruses have been reported previously. Cheevers et al. (10) and Ishizaki et al. (22) analyzed EIAV polypeptide composition by SDS-PAGE and reported two (p29 and p13) and three (p25, p14, and p11) major non-glycosylated structural proteins, respectively. Examinations of the polypeptides of visna virus, the prototype lentivirus, similarly revealed two (7) or three (27, 42) major non-glycosylated proteins. Based on these observations, it has been suggested that polypeptide composition may serve as a distinguishing feature between the lentivirus and oncovirus subfamilies of the Retroviridae (30). However, we reported previously (38) that EIAV contains four major non-glycosylated proteins, apparently analogous to those found in prototype C oncoviruses. The experiments described here confirm the biochemical and serological uniqueness of each EIAV protein. Thus, our findings are in agreement with those of Charman et al. (8), who resolved four structural components (p26, p12, p10 [acidic], and p10 [basic]) by isoelectric focusing techniques.

The biochemical properties of the EIAV proteins are strikingly similar to those described previously for avian and murine oncoviruses and thus suggest certain locations for the structural proteins in intact virions. EIAV p26 appears to be the major internal component analogous to avian virus p27 and murine virus p30, whereas EIAV p11 constitutes the basic ribonucleoprotein like avian virus p12 and murine virus p10. Our results also indicate that EIAV p15 is a phosphoprotein. Based on the presence of phosphoamino acids and, perhaps, an affinity for viral RNA, EIAV p15 may be the analog of avian virus p19 and murine virus p12. However, further experimentation will be required to support this hypothesis. Finally, EIAV p9, an acidic virion component, may perform a role similar to that of avian or murine virus p15. Thus, together with previous data (38), these results support the concept that lentiviruses and oncoviruses indeed have similar patterns of polypeptide composition and organization. Moreover, the development of efficient procedures for antigen purification, biochemical characterization, and serological analysis of EIAV polypeptides has provided the tools necessary for comparing viral isolates from sequential clinical episodes in infected animals. Such comparisons may provide information concerning the mechanism of viral persistence in equine infectious anemia.

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